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Anti-inflammatory effects of red pepper (*Capsicum baccatum*) on carrageenan- and antigen-induced inflammation

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Abstract

Inflammation is a pivotal component of a variety of diseases, such as atherosclerosis and tumour progression. Various naturally occurring phytochemicals exhibit anti-inflammatory activity and are considered to be potential drug candidates against inflammation-related pathological processes. *Capsicum baccatum* L. var. *pendulum* (Willd.) Eshbaugh (Solanaceae) is the most consumed species in Brazil, and its compounds, such as capsaicinoids, have been found to inhibit the inflammatory process. However, the anti-inflammatory effects of *C. baccatum* have not been characterized. Thus, this study was designed to evaluate the effects of *C. baccatum* juice in animal models of acute inflammation induced by carrageenan and immune inflammation induced by methylated bovine serum albumin. Pretreatment (30 min) of rats with pepper juice (0.25–2.0 g kg^{-1}) significantly decreased leucocyte and neutrophil migration, exudate volume and protein and LDH concentration in pleural exudates of a pleurisy model. This juice also inhibited neutrophil migration and reduced the vascular permeability on carrageenan-induced peritonitis in mice. *C. baccatum* juice also reduced neutrophil recruitment and exudate levels of pro-inflammatory cytokines TNF-α and IL-1β in mouse inflammatory immune peritonitis. Furthermore, we demonstrated that the main constituent of *C. baccatum* juice, as extracted with chloroform, is capsaicin. In agreement with this, capsaicin was able to inhibit the neutrophil migration towards the inflammatory focus. To our knowledge, this is the first demonstration of the anti-inflammatory effect of C. *baccatum* juice and our data suggest that this effect may be induced by capsaicin. Moreover, the anti-inflammatory effect induced by red pepper may be by inhibition of pro-inflammatory cytokine production at the inflammatory site.

Introduction

Red peppers have been used for several thousand years as food additives and for a broad variety of medical applications in Indian, Native American, African and Chinese medical traditions (Govindarajan & Sathyanarayana 1991; Szallasi & Blumberg 1999). The red pepper *Capsicum baccatum* L. var. *pendulum* (Willd.) Eshbaugh (Solanaceae) is known popularly in Brazil as pimenta dedo-de-moça and it is the most consumed species in Brazil, mainly in South and Southeast regions (Linguanotto 2004).

Red peppers have been claimed to enhance immune response, act in an anti-inflammatory manner, lower blood pressure, reduce excessive blood clotting and reduce blood sugar levels, but no formal examination of these claims has been published (Surh & Lee 1995, 1996). Capsaicin is the pungent component of red peppers and because of its analgesic and anti-inflammatory activity has been used in clinical practice. Thus, topical application of capsaicin has a therapeutic value in a variety of neuropathic pain conditions, including rheumatoid arthritis, osteoarthritis, diabetic neuropathy, postmastectomy pain syndrome, psoriasis, burning mouse syndrome and herpes zoster (Group T.S.C. 1991; McCarthy & McCarty 1992).

Chemically, capsaicin is a derivative of vanillyl amide (8-methyl-*N*-vanillyl-6-nonenamide) and has a molecular weight of 305.42 Da. With respect to potency, target and mechanism of action, capsaicin is known to have two kinds of effects—a brief stimulation of primary afferent nerve fibres followed by desensitization to capsaicin and a cell non-selective effect.

This nonselective effect does not have any long-lasting consequence on the responding cells (Holzer 1991). The capsaicin receptor, vanilloid receptor 1 (TRPV1), has been shown to be highly expressed by nociceptive neurons in dorsal root and trigeminal ganglia (Caterina et al 1997, 2000). Capsaicin binds to TRPV1 on sensory neurons to convey the sensation of pain. Apart from its neurological functions, capsaicin has also been shown to be immunologically active in generating more antibody-producing cells compared with untreated controls. Dietary capsaicin in mice has been shown to enhance lymphocyte proliferation and serum immunoglobulin levels (Yu et al 1998). Inhalation of capsaicin has been shown to interfere with neural responses involved in inflammation of Lewis rat lungs, and such interference modulates immunity to inhaled antigens (Kradin et al 1997). Moreover, capsaicin inhibited the development of paw inflammation induced by carrageenan in rats (Manjunatha & Srinivasan 2006). Thus, this study was designed to evaluate the effects of *Capsicum baccatum* juice in animal models of acute and immune inflammation induced by carrageenan and methylated bovine serum albumin, respectively. This spice was chosen since it is common in Brazilian gastronomic use, yet its anti-inflammatory effect has not been characterized.

Materials and Methods

Drugs and *Capsicum baccatum* juice preparation

Carrageenan, methylated bovine serum albumin (mBSA), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Evans Blue and May–Grunwald–Giemsa stain and capsaicin (from *Capsicum* sp. ≥ 95%) were purchased from Sigma Chemical Company (St Louis, MO). The fruits of red pepper *C. baccatum* var. *pendulum* were collected from plants cultivated in the Pontifícia Catholic University of the Rio Grande do Sul (PUCRS), Brazil, cleaned and the seeds were withdrawn. The pepper fruits (without seeds) were pressed in a garlic press and the juice obtained was filtered with a 0.45 μm GD/X disposable syringe filter (Fisher Scientific, Pittsburgh, PA). Each gram of red pepper produced 100 μL of juice.

Animals

Male *Rattus norvegicus* (Wistars, 200–250 g) or C57BI/6 mice (18–20 g) were used. Animals were kept in appropriate cages, with a 12-h dark–light cycle in a temperature-controlled room. Animals had free access to water and food and were kept in the laboratory about 7 days for acclimatization before the experiment. All animals were manipulated in accordance with the Guiding Principles in The Care and Use of Animals, approved by the Council of the American Physiologic Society. This study was approved by PUCRS Ethic Committee (06/02971, 2005).

Red pepper juice treatment

Rats were pretreated 30 min with red pepper juice before carrageenan administration according to the following protocol: for the doses of 0.25, 0.5 and 2 g kg^{-1} juice volumes of ~6.25, 12.5 and 50 μL were all diluted to a final volume of 200 μL, respectively, with saline. In similar fashion, mice were pretreated with red pepper juice 30 min before carrageenan administration in the following protocol: for the doses of 0.20, 2.0 and 20 g kg^{-1} juice volumes of ~0.40, 4.0 and 40 μL were all diluted to a final volume of 200 μL, respectively, with saline.

Carrageenan-induced pleurisy

Rats were pretreated (30 min) with 200 μL intraperitoneal saline or *C. baccatum* juice (0.25, 0.5, 1 or 2 g kg^{-1}). Subsequently, rats were anaesthetized with ketamine–xylasine (3:1, 2 mL kg^{-1}) and 200 μg/0.2 mL carrageenan suspended in saline, or 0.2 mL saline, was injected into the pleural cavity, as described previously (Alves Filho et al 2004). Four hours after carrageenan administration the rats were euthanized in an atmosphere of CO_2. The chest was carefully opened and the pleural cavity was washed with 2 mL of sterile saline solution (NaCl 0.9%) containing 1 mM EDTA. The exudate and washing solution were removed by aspiration and data were analysed. Exudates contaminated with red blood cells were rejected (Lunardelli et al 2006).

Carrageenan-induced peritonitis

Mice were pretreated (30 min, s.c.) with 200 μL of saline, *C. baccatum* juice (0.2, 2 or 20 g kg^{-1}) or capsaicin (2 mg kg^{-1}). The peritonitis was induced by intraperitoneal carrageenan administration (500 μg/500 μL). Six hours after carrageenan administration the mice were euthanized in an atmosphere of CO_2, and the peritoneal cavity was washed with 3 mL of phosphate-buffered saline (PBS) containing 1 mM EDTA over a period of 3 min and the solution was then recovered.

Immunization procedure

C57Bl/6 mice were immunized. Briefly, on day 0, the mice received a single subcutaneous injection of a proteic antigen mBSA (500 μg) in 0.2 mL of an emulsion containing 0.1 mL of saline and 0.1 mL of CFA. Mice received booster injections of mBSA dissolved in IFA on days 7 and 14. Non-immunized (NI) mice were given similar injections but without the antigen (mBSA). Twenty-one days after the initial injection, the immunized and NI mice were challenged with mBSA (30 μg/cavity, i.p.), or only saline in immunized mice (control).

Leucocyte and neutrophil count

Total leucocyte count was obtained with a cell counter (Coulter Analyzer, Ac T Series; Coulter Corp., Miami, FL) and differential cell counting was carried out on cytocentrifuge slides (Cytospin 3; Shandon Southern Products, Atsmoore, UK) with the May–Grunwald–Giemsa stain. Data are reported as means ± standard error of the mean (s.e.m) per cavity and each count was carried out at least twice, with differences of less than 10%.

Exudate volume on pleural exudate

The exudate volume was measured and results were expressed by subtracting the volume (2 mL of PBS) injected in the pleural cavity from total volume recovered.

Protein concentration in pleural exudate

The plasma exudation was determined by protein concentration in exudate. The pleural exudate recovered from the pleural cavity was centrifuged (1200 *g*) for 10 min and the total protein content of the supernatant quantified spectrophotometrically using the Biuret method (Weichselbaum 1946).

Lactate dehydrogenase (LDH) level

LDH level in the pleural exudate was analysed by commercial LDH kit purchased from Labtest Diagnostic system (Porto Alegre, RS, Brazil). The measurements were performed in duplicate, varied ≤ 10% and were carried out at the same time.

Vascular permeability

The vascular permeability was analysed by Evans Blue Test, as described previously (Thurston et al 2000). Thirty minutes before carrageenan administration, Evans blue (50 mg kg^{-1}) was injected with 100 μL of saline intravenously into the ocular plexus. Mice were euthanized in an atmosphere of CO_2 6 h after carrageenan administration and the peritoneal cavity was washed with 3 mL of PBS. Evans Blue content was calculated using an Evans blue standard and the absorbance of each sample was measured at 620 nm using a spectrophotometer.

Determination of TNF-α and IL-1β levels

TNF-α and IL-1β levels in the exudates from antigen-challenged immunized mice were detected by ELISA (Taktak & Lee 1991). Briefly, microtitre plates were coated overnight at 4°C with an immunoaffinity-purified polyclonal sheep antibody against TNF-α (2 μg mL^{-1}) or IL-1β (2 μg mL^{-1}). After blocking the plates, recombinant murine TNF-α or IL-1β standards at various dilutions and the samples were added in duplicate and incubated overnight at 4°C. Rabbit biotinylated immunoaffinity-purified pAb anti-TNF-α (1:500) or anti-IL-1β (1:1000) was added, followed by incubation at room temperature for 1 h. Fifty microlitres of avidin-HRP (1:5000 dilution; DAKO A/S, Denmark) was added to each well; after 30 min, the plates were washed and the colour reagent OPD (200 μg/well; Sigma) was added. After 15 min, the reaction was stopped with 1 M H_2SO_4 and the optical density (O.D.) measured at 490 nm. The results were expressed as pg/mL of TNF-α and IL-1β, based on the standard curves.

Gas chromatography analyses

Three grams of the juice from *C. baccatum* were incubated with 10 mL of chloroform for 12 h under gentle agitation at room temperature and the sample was passed through a 0.45 μmGD/X disposable syringe filter. Then, 2 mL of this sample were dried with liquid nitrogen and reconstituted with 0.1 mL of chloroform, and 1 μL of this solution was injected into a gas chromatographer (GC) (Shimadzu, QP2010). GC separations were accomplished using a 30 m × 0.25 mm (DB-5MS) capillary column with 0.25 μm film thickness (HP-1). Operating conditions were 240°C for injector and 100°C for column oven, and the carrier gas (H_2) flow rate was 1.20 mL min^{-1}. The capsaicin peak was detected by comparison with the capsaicin standard retention time (19.396 min). The capsaicin standard was dissolved in chloroform for GC analysis.

Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 11.0. Data were analysed for significance using one-way analysis of variance. The Bonferroni's post-hoc testing was used for group comparisons and $P < 0.05$ was considered significant. Data are reported as mean ± s.e.m., and are representative of two or three independent experiments with 5 or 6 animals in each group.

Results

Administration of carrageenan (200 μg/200 μL/cavity, i.pl.) to rats pretreated (30 min, i.p., n = 6) with saline induced a significant ($P < 0.05$) amount of leucocyte migration (46.27 ± 0.74 leucocyte × 10^6/cavity), exudate volume (0.93 ± 0.04 mL) and protein content (1.70 ± 0.03 mg mL^{-1}) compared with the control group pretreated intraperitoneally with saline and injected intrapleurally with saline (n = 6; 10.57 ± 1.04, 0.07 ± 0.02 and 0.16 ± 0.04, for leucocyte, exudate volume and protein content, respectively). To choose a functional dose of *C. baccatum* juice, the rats were pretreated (30 min, i.p., n = 6 per group) with 0.25, 0.5 or 2 g kg^{-1} of this juice. Pretreatment of rats with 2 g kg^{-1} significantly ($P < 0.05$) reduced carrageenan-induced leucocyte migration (25.71 ± 2.67), exudate volume (0.46 ± 0.03) and protein content (1.06 ± 0.10) as compared with rats pretreated with saline and intrapleural injections of carrageenan. Therefore, 2 g kg^{-1} was selected as the optimal dose for detection of anti-inflammatory effects of *C. baccatum* juice in the pleurisy model. The results for groups pretreated with 0.25 g kg^{-1} and 0.5 g kg^{-1} *C. baccatum* juice were 39.00 ± 4.38 and 41.40 ± 5.57, 0.65 ± 0.11 and 0.63 ± 0.04, and 1.17 ± 0.18 and 1.13 ± 0.07 for leucocyte, exudate volume and protein content, respectively.

In this context, Table 1 confirms that the pretreatment of rats with 2 g kg^{-1} of *C. baccatum* juice significantly reduced carrageenan-induced leucocyte migration, volume of exudates and protein content. Moreover, Table 1 shows that this treatment also significantly reduced the neutrophil content and exudate LDH release ($P < 0.05$).

To confirm the anti-inflammatory effect of *C. baccatum* juice, it was tested in the peritonitis model induced by carrageenan. Carrageenan induced a significant neutrophil migration to the murine peritoneal cavity (Figure 1A, $P < 0.05$) and this neutrophil migration was inhibited by pretreatment (30 min, s.c.) of the mice with 2 g kg^{-1} or 20 g kg^{-1} of *C. baccatum* juice. In addition to neutrophil migration, vascular leakage is a major parameter in inflammation. Therefore, we tested the effect of *C. baccatum* juice in the carrageenan-induced microvascular permeability. Figure 1B shows that pretreatment (30 min, s.c.) with 20 g kg^{-1} of *C. baccatum* juice significantly decreased ($P < 0.05$) the content of Evans blue in peritoneal fluid. In another set of experiments, the neutrophil migration induced by mBSA in immunized mice was inhibited by *C. baccatum* in a dose-dependent manner (Figure 2A). Supporting

Table 1 Anti-inflammatory effect of *C. baccatum* extract on carrageenan-induced pleurisy in rats

Groups	Leucocytes (cells × 10^6/cavity)	Neutrophils (cells × 10^6/cavity)	Exudate (mL)	Total protein (mg mL^{-1})	LDH (U L^{-1})
Saline i.p. plus saline i.pl.	12.25 ± 1.41	2.94 ± 0.43	0.17 ± 0.04	2.03 ± 0.10	28.85 ± 3.10
Pepper i.p. plus saline i.pl.	10.50 ± 1.41	4.93 ± 1.54	0.15 ± 0.05	2.58 ± 0.60	33.28 ± 1.49
Saline i.p. plus carrageenan i.pl.	46.25 ± 9.08*	36.53 ± 8.31*	0.92 ± 0.22*	7.60 ± 0.87*	48.51 ± 3.24*
Pepper i.p. plus carrageenan i.pl.	20.00 ± 3.09#	15.16 ± 2.87#	0.07 ± 0.06#	4.54 ± 0.50#	29.98 ± 2.02#

Rats were pretreated with intraperitoneal (i.p., 30 min) saline or *C. baccatum* juice (pepper, 2.0 g kg^{-1}) and received an intrapleural (i.pl.) injection of saline or carrageenan (200 μg/200 μL). These parameters were analysed 4 h after induction of pleurisy. Data were expressed as means ± s.e.m. *$P < 0.05$ vs saline i.p. plus saline i.pl. #$P < 0.05$ vs saline i.p. plus carrageenan i.pl.

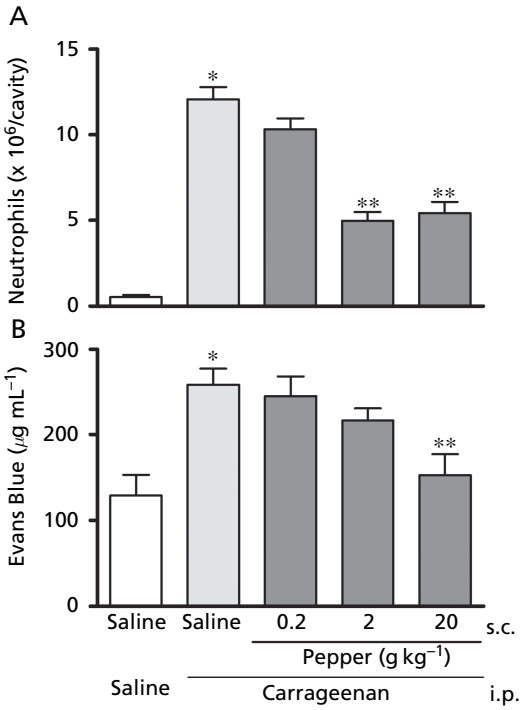

Figure 1 Anti-inflammatory effect of *C. baccatum* juice on carrageenan-induced peritonitis in mice. *C. baccatum* juice reduces neutrophil migration (A) and vascular permeability (B) on carrageenan-induced peritonitis in mice. Mice were pretreated subcutaneously (s.c., 30 min) with saline (Control) or *C. baccatum* juice (Pepper, 0.20–20.0 g kg^{-1}) and received an intraperitoneal (i.p.) injection of carrageenan (500 μg/500 μL). Six hours after induction of peritonitis, mice were euthanized and the peritoneal cavities were washed with 3 mL of sterile PBS solution containing 1 mM EDTA and data were analysed. Data are expressed as mean ± sem. *$P < 0.05$ vs saline s.c. + saline i.p. **$P < 0.05$ vs saline s.c. + carrageenan i.p.

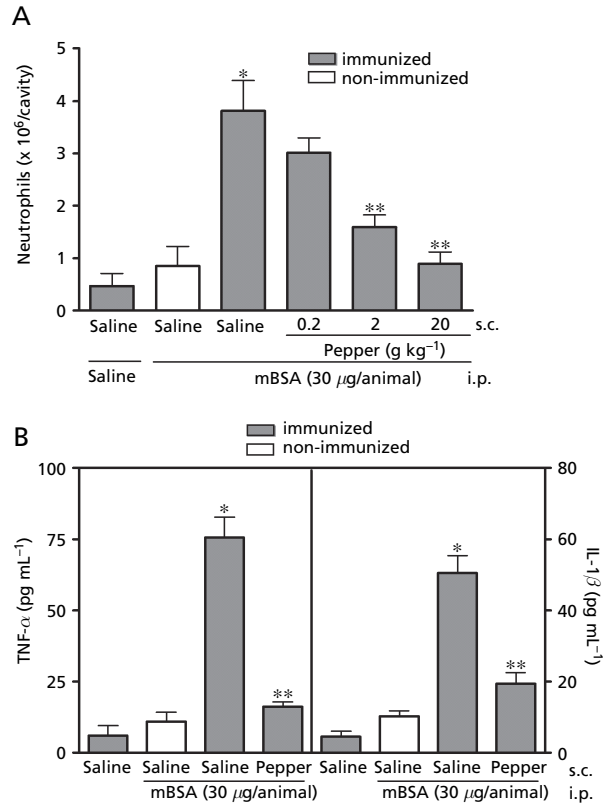

Figure 2 Anti-inflammatory effect of *C. baccatum* juice on immune inflammation induced by mBSA. *C. baccatum* juice reduces neutrophil migration (A) and production of TNF-α and IL-1β (B) on mBSA-induced immune peritonitis in mice. Immunized mice were pretreated subcutaneously (s.c., 30 min) with *C. baccatum* juice (Pepper) at indicated doses or saline and then challenged with intraperitoneal (i.p.) administration of mBSA. The peritoneal exudate was harvested 4 h after saline or mBSA-challenge and the number of neutrophils and concentration of TNF-α and IL-1β were determined. Data are mean ± s.e.m., n = 5. *$P < 0.05$ vs saline s.c. + saline i.p. or saline s.c. + mBSA i.p. (non-immunized mice); **$P < 0.05$ vs saline s.c. + mBSA i.p. (immunized mice).

the anti-inflammatory effects of *C. baccatum* in antigen-induced inflammation, we observed that the concentrations of the pro-inflammatory mediators TNF-α and IL-1β in the peritoneal fluid of mice challenged with mBSA were decreased (Figure 2B). The immunization state of the mice was confirmed, since immunized mice had higher titres of serum IgG against mBSA than non-immunized mice (data not shown).

To identify the possible constituents of the *C. baccatum* juice, extraction was performed with chloroform and separation by GC as described in Materials and Methods. Figure 3A

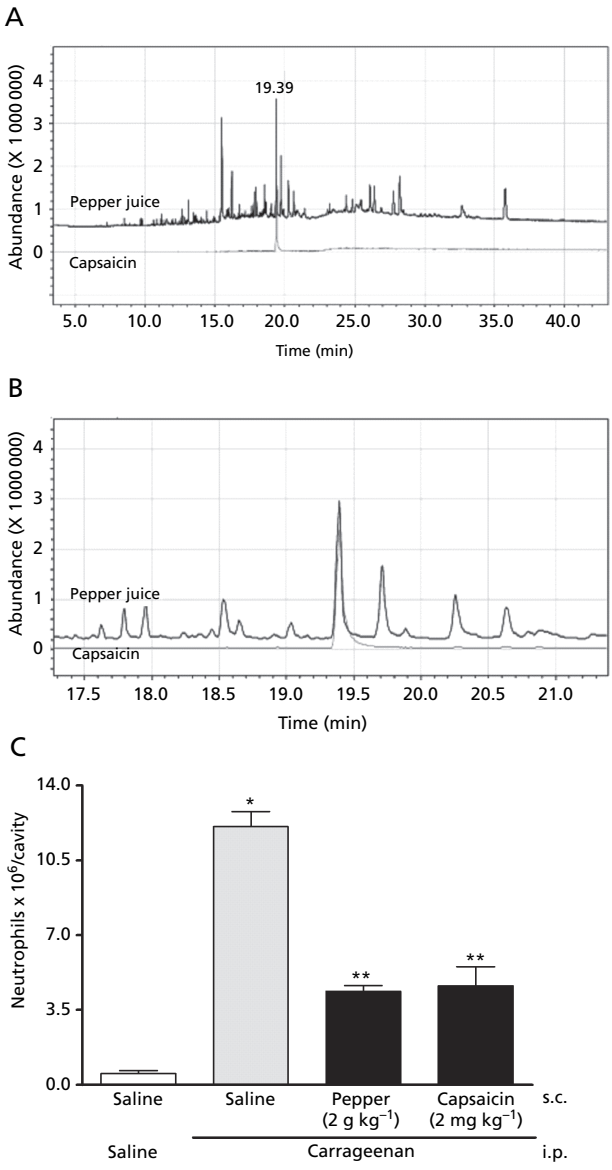

Figure 3 A. Gas chromatograph analyses. Total ion current traces obtained by injection of 1.0 μL of the extract from *C. baccatum* juice (see Methods) and by 1.0 μL of the capsaicin standard. The data are expressed as relative abundance (× 1 000 000) of these ions. B. Total ion current traces of the same samples shown in A; however, between the times 17.5 and 21.0 min (retention time of the capsaicin standard was 19.39 min). C. Capsaicin reduces the neutrophil migration on carrageenan-induced peritonitis in mice. Mice were pretreated subcutaneously (s.c., 30 min) with saline (Control), *C. baccatum* juice (Pepper 2.0 g kg^{-1}) or capsaicin (2 mg kg^{-1}) and received an intraperitoneal (i.p.) injection of carrageenan (500 mg/500 μL). Six hours after induction of peritonitis, the neutrophils were counted in the peritoneal cavities. Data were expressed as means ± sem. $^{*}P < 0.05$ vs saline s.c. + saline i.p. $^{**}P < 0.05$ vs saline s.c. + carrageenan i.p.

shows a typical chromatogram of the total ions for a red pepper extract sample (upper line) and for a standard sample of capsaicin (lower line). The analyses of the total ions of the red pepper extract showed a major peak with a retention time of 19.392 min. This peak represents 10.94% of the total ions and has a similar retention time to the capsaicin standard (19.396 min). Figure 3B shows an enlargement of this chromatogram between 17.5 and 21.0 min. To confirm that capsaicin may be involved in the anti-inflammatory effect of *C. baccatum* juice, the mice were pretreated subcutaneously with capsaicin (2 mg kg^{-1}) and the neutrophil migration to the inflammatory focus was analysed. Figure 3C shows that capsaicin significantly inhibited neutrophil migration to the peritoneal cavity. These experiments strongly suggest that capsaicin is one of the major compounds of *C. baccatum* extract and may be involved in the inhibitory effect of *C. baccatum* juice.

Discussion

The inflammatory response is orchestrated by a large range of mediators able to promote vascular events, oedema and recruitment of inflammatory cells (Calixto et al 2003). Several spices and their compounds, such as polyphenols, ascorbic acid and capsaicinoids, have been found to inhibit the inflammation process as well as tumorigenesis in experimental animals (Yoon & Baek 2005), and are considered to be potential drug candidates against the inflammation-related pathological processes, (Calixto et al 2003). In this context, this study demonstrates for the first time the anti-inflammatory effect of *C. baccatum* var. *pendulum* juice. The pretreatment of rats with *C. baccatum* juice decreased neutrophil migration, exudate volume, protein content and LDH concentration in pleural exudate. This juice also inhibits neutrophil recruitment and reduces the vascular permeability in carrageenan-induced peritonitis in mice. In the same way, the pretreatment with *C. baccatum* juice inhibits the neutrophil migration to the peritoneal cavity and reduces significantly the levels of pro-inflammatory cytokines TNF-α and IL-1β in peritoneal fluid of immunized mice. Furthermore, our data strongly suggest that capsaicin is one of the major compounds present in *C. baccatum* juice and may be involved in its anti-inflammatory activity.

Red peppers are common in Brazilian gastronomy and are consumed in-natura or conserved in oil. *C. baccatum* is the most consumed species in Brazil and concentrations of total phenols, capsaicinoids and ascorbic acid were significantly greater in *C. baccatum* fruit compared with other species of peppers such as *C. annuum* or *C. frutescens* (Antonious et al 2006). These compounds of red pepper exhibit anti-inflammatory or anti-oxidant properties (Carr & Frei 1999; Kim et al 2003; Chen et al 2006). In this context, we showed that juice obtained from *C. baccatum* had in-vivo anti-inflammatory effects, and these effects, probably, were induced by capsaicin. In agreement with our data, Kim et al (2003) demonstrated that capsaicin inhibits the production of pro-inflammatory mediators such as PGE_2 and nitric oxide by nuclear transcription factor kappa B (NF-κB) inactivation in murine peritoneal macrophages. Interestingly, the inhibitory action of capsaicin on the release of pro-inflammatory molecules was not mediated by TRPV1 (Kim et al 2003), which is a specific receptor for capsaicin, indicating the involvement of an alternative mechanism. In this context, the same group demonstrated that capsaicin suppresses the production of TNF-α by acting as an agonist for

PPARγ in LPS-stimulated murine RAW 264.7 macrophages (Park et al 2004). In this context, neutrophil migration observed in models of immune inflammation is mediated by release of TNF-α and IL-1β (Ramos et al 2005; Nambu et al 2006) and the pretreatment with *C. baccatum* juice inhibits the levels of TNF-α and IL-1β in immunized mice. These data suggest that the in-vivo anti-inflammatory effect of this juice may be by inhibition of pro-inflammatory cytokine production at inflammatory sites. In addition to capsaicinoids, ascorbic acid and phenols have important roles in the inflammatory process, mainly due to their antioxidant properties (Carr & Frei 1999; Surh 2002). Therefore, the anti-inflammatory effect of *C. baccatum* juice may be a consequence of these antioxidants compounds. Despite these data showing the anti-inflammatory effects of red pepper and capsaicin, there are no epidemiologic studies showing whether red pepper intake may be beneficial in human inflammatory diseases.

Conclusion

Data from this study show for the first time the anti-inflammatory activity of *Capsicum baccatum L.* var. *pendulum* (Willd.) Eshbaugh juice and suggest that this effect may be induced by capsaicin. Moreover, the anti-inflammatory effect of this red pepper may be by inhibition of pro-inflammatory cytokine production at the inflammatory site. This juice contains different molecules with antioxidant or anti-inflammatory properties and could be employed for new drug development.

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